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The Antisense Oligonucleotide ISIS 2922 Prevents Cytomegalovirus-Induced Upregulation of IL-8 and ICAM-1 in Cultured Human Fibroblasts

Jindrich Cinatl, Jr.,[1]* Ruslan Kotchetkov,[1] Eveline Weimer,[1,3] Roman A. Blaheta,[1,2] Martin Scholz,[1] Jens-Uwe Vogel,[1,2] Herman O. Gümbel,[3] and Hans Wilhelm Doerr[1]

[1]*Zentrum der Hygiene, Institut für Medizinische Virologie, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany*
[2]*Zentrum für Kinderheilkunde und Jugendmedizin, Abteilung Pädiatrische Hämatologie und Onkologie Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany*
[3]*Zentrum für Augenheilkunde, Johann Wolfgang Goethe-Universität, Frankfurt am Main, Germany*

Human cytomegalovirus (HCMV) infection is associated with excessive proinflammatory immune responses such as cytokine/chemokine production or upregulation of adhesion molecules on the host cells. It is assumed that these features of HCMV-related immunopathology can not be treated effectively with currently available anti HCMV drugs. In the present study the efficacy of ganciclovir (GCV), foscarnet (PFA), cidofovir (HPMPC), and ISIS 2922, an antisense oligonucleotide complementary to HCMV immediate-early (IE) mRNA, was investigated on HCMV-induced secretion and functional activity of the C-X-C chemokine IL-8 and the expression of the intercellular adhesion molecule-1 (ICAM-1). As compared with mock-infected cells IL-8 production was increased up to 9-fold and ICAM-1 expression up to 4-fold in HCMV-infected fibroblasts. Treatment of infected cells with GCV (40 μM), PFA (200 μM) or HPMPC (2 μM) suppressed completely virus replication as demonstrated by quantification of late (L) antigen expression and infectious virus production. These drugs, however, failed to inhibit IE antigen expression and did not prevent HCMV-induced upregulation of IL-8 and ICAM-1. In contrast, ISIS 2922 (1 μM) suppressed both IE and L antigen expression by 99% and inhibited infectious virus production by 10^4-fold. Moreover, ISIS 2922 significantly suppressed HCMV-induced upregulation of both IL-8 and ICAM-1 expression on the transcriptional and on the protein level. Our results indicate that ISIS 2922 but not inhibitors of HCMV DNA prevents HCMV-induced upregulation of IL-8 and ICAM-1, both hallmarks of inflammatory processes. Thus, inhibition of HCMV IE expression with ISIS 2922 may be an important strategy for the treatment of HCMV-related immunopathogenesis. ***J. Med. Virol. 60:313–323, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: human cytomegaloviruses; chemoattractants; antiviral drugs; immunopathology; antisense oligonucleotides

INTRODUCTION

Human cytomegalovirus (HCMV) is a significant pathogen in immunocompromised patients such as transplant recipients and AIDS patients. These patients suffer from severe manifestations such as graft rejection [von Willebrand et al., 1986; Lautenschlager et al., 1997; Pouteil Noble et al., 1993], pneumonia [Emanuel et al., 1988] or HCMV retinitis [Holland et al., 1983]. Numerous pathogenic changes in infected organs stem from virus replication in permissive cells resulting in their lysis. On the other hand, HCMV may alter the expression of genes coding for proteins with proinflammatory activity probably without the requirement for virus replication [Craigen and Grundy, 1996; Scholz et al., 1998]. HCMV infection has been associated with increased cytokine production, directly by the infected cells or indirectly by immune cells that interact with infected cells [Scholz et al., 1995; Grundy, 1998]. A number of HCMV-induced cytokines including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interferon-α (IFN-α) possess proinflammatory activity [St. Jeor et al., 1993; Grundy, 1998].

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*Correspondence to: Dr. Jindrich Cinatl, Institut für Medizinische Virologie, Zentrum der Hygiene, Klinikum der Johann Wolfgang Goethe-Universität, Sandhofstr. 2-4, D-60528 Frankfurt am Main, Germany. E-mail: cinatl@em.uni-frankfurt.de

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Because HCMV has been associated with inflammatory disorders such as graft rejection crises after organ transplantation it is assumed that HCMV triggers leukocytic infiltration of infected tissues. In vitro experiments performed in different laboratories have shown that HCMV induced the upregulation of several adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) or lymphocyte function-associated molecule-3 (LFA-3) [Scholz et al., 1992; Grundy and Downes, 1993]. Moreover, HCMV infection was reported to be associated with increased functional binding of leukocytes to infected cells that is a major step in leukocyte transmigration [Span et al., 1991; Tuder et al., 1991; Grundy et al., 1993; Blaheta et al., 1994]. Recently, it has been shown that HCMV induces enhanced production of both C-X-C (i.e., IL-8 and GRO-α) and C-C (i.e., RANTES and MIP-1α) chemokines in different cell types [Michelson et al., 1997; Muryama et al., 1997; Grundy et al., 1998; Scholz et al., 1998]. Importantly, secretion of C-X-C chemokines by HCMV-infected endothelial cells resulted in increased transendothelial neutrophil migration [Craigen et al., 1997]. HCMV-induced chemoattraction of leukocytes in concert with enhanced expression of adhesion molecules may result in unspecific infiltration of the infected tissue and thus leads to HCMV-associated immunopathogenic effects.

It has been shown previously that a variety of HCMV-induced alterations in cellular characteristics including expression of adhesion molecules and synthesis of cytokines or chemokines occur early after virus infection and may result from the transactivation activity of immediate early (IE) proteins [Grundy et al., 1998; Scholz et al., 1998; Burns et al., 1999]. The antiviral agents currently in clinical use, e.g. ganciclovir (GCV), foscarnet (PFA) and cidofovir (HPMPC) exert their actions by preventing viral DNA replication, and the subsequent synthesis of HCMV late proteins [Vogel et al., 1998]. These drugs prevent efficiently virus replication but have no influence on events, e.g., IE expression, that occur before viral DNA synthesis [Cinatl et al., 1999]. Thus, treatment with inhibitors of viral DNA synthesis may be insufficient to prevent these pathogenic features of HCMV infection. To develop novel strategies for the treatment of HCMV disease we investigated the ability of antiviral drugs to impair HCMV-related features of immunopathogenesis. In this report we present our in vitro data on the efficacy of the HCMV DNA inhibitors GCV, PFA, and HPMPC and of the HCMV IE mRNA inhibitor ISIS 2922 to suppress HCMV-induced IL-8 and ICAM-1 expression on the transcriptional and protein level. IL-8 and ICAM-1 were selected for these experiments because during inflammation they represent major hallmarks of neutrophil activation and intercellular adhesion/transmigration [Harada et al., 1996; van de Stolpe and van der Saag, 1996].

MATERIALS AND METHODS

Cell Culture

HFF cells were established in our laboratory as described previously [Cinatl et al., 1995]. Cells were grown in a culture medium composed of Eagle's MEM supplemented with 20% FBS. After reaching confluence, cells were subcultured at 6-day intervals. HFF cells between 3 and 6 subcultures were used in the experiments.

Virus Preparation

The HCMV laboratory strain AD169 was obtained from ATCC. Virus stock was prepared in HFF cells incubated in MEM supplemented with 4% FBS. The respective titres were determined by examination of immediate early forming units (I.E.F.U.) in HFF cells as described previously [Cinatl et al., 1995]. Mock-infected inocula were prepared in an identical fashion, except that cell monolayers were not infected with HCMV.

Inactivation of virus was carried out by exposure of virus solution to UV light (220 V, 12 W) for 15 min [Muryama et al., 1997]. Samples of irradiated virus were then used to infect HFF. UV-irradiated samples were free of infectious virus as detected by plaque titration.

Filtered virus inocula (virus free supernatants) were prepared by filtering virus stocks through a Microsep microconcentrator with a cutoff at 300,000 molecular weight (Filtron Technology Corp., Northborough, MA) at 3,000 × *g* for 12 hr at 4°C. The filtrate collected from the bottom of the filter apparatus was added to HFF. The filtrate samples were free of infectious virus as demonstrated by plaque titration.

Antiviral Drugs

GCV (Hoffman-La Roche AG, Grenzach-Wyhlen, Germany) was prepared freshly (on the day of each experiment) in distilled water. Foscarnet (PFA; Sigma, Deisenhofen, Germany) and HPMPC (cidofovir, Pharmacia & Upjohn, Erlangen, Germany) were dissolved in distilled water and stored at aliquots at –20°C. ISIS 2922, a phosphorothioate oligonucleotide that is complementary to HCMV IE mRNA and noncomplementary control oligonucleotide ISIS 26062, were kindly provided by Isis Pharmaceuticals, Inc. (Carlsbad, CA). Both ISIS 2922 and ISIS 26062 were dissolved in phosphate buffered saline (PBS) at a concentration of 10 mM and aliquots were stored at –20°C until use. To enhance oligonucleotide uptake ISIS 2922 or ISIS 26062 were complexed to cationic liposomes (DOTAP; Boehringer Mannheim, Germany) immediately before virus infectivity assay. The mixing of oligonucleotides with DOTAP was performed according to the manufacturer's instructions.

Antiviral Assay

Confluent cultures of HFF cells were incubated with HCMV infectious strains at a multiplicity of infection

(MOI) of 1 or with UV-inactivated samples or with filtered virus inocula. After incubation for 1 hr required for virus adsorption, the cells were washed with phosphate-buffered saline and incubated in culture medium containing 2% FBS. In some experiments cells incubated with infectious virus were treated with antiviral agents GCV, PFA, HPMPC or antisense oligonucleotides ISIS 2922 or ISIS 26062. Cells were treated with ISIS 2922 according to established protocols [Azad et al., 1993; Anderson et al., 1996]. Briefly, the cell monolayers were pretreated with ISIS 2922 overnight in MEM containing 0.2% FBS and then washed three times with PBS before infection with HCMV at MOI of 1. After 1 hr of incubation, virus was removed and fresh medium containing test compounds was added. To avoid differences in the treatment procedure the cells were incubated with GCV, PFA or HPMPC in the same way as described for ISIS 2922. The number of cells producing viral antigens were examined 24 hr and 72 hr after infection by immunoperoxidase staining. Immediate early antigens (IEA) were detected using the monoclonal antibody Mab810 (Chemicon, Hofheim, Germany) that binds to a shared epitope at the aminotermini of the IE55, IE86, and IE72 proteins. Late antigens (LA) were detected using the monoclonal antibody directed against 67-kDa LA (DuPont, Bad Homburg, Germany).

Effects of antiviral drugs on virus yield in HFF cells were measured by a method described previously [Cinatl et al., 1995]. Briefly, HFF were preincubated (overnight) with antiviral drugs and then washed three times with PBS before infection with HCMV at MOI 1. After 90 min incubation cells were washed with PBS and medium containing 4% FBS without or with different concentrations of antiviral drugs was added. Virus yield was determined in a single-cycle assay format (i.e., 3 days after infection) using titration in HFF monolayers and expressed as I.E.F.U./ml.

IL-8 Assay

IL-8 protein levels were quantified by immunoassay using commercially available ELISA kits according to the manufacturer's instructions (R&D Systems, Wiesbaden-Nordenstadt, Germany). Cytokine levels were determined in supernatant fluid harvested from mock- or HCMV-infected cultures at various times post infection and stored at −80°C before being assayed. IL-8 levels were expressed as ng of IL-8 per 10^6 cells.

ICAM-1 Measurement

To investigate the expression of ICAM-1, 5×10^5 mock- or HCMV-infected cells at different times post-infection were fixed for 10 min in 4% buffered formaldehyde. After washing twice in buffer containing 0.5% Tween-20, cells were incubated for 30 min with mouse monoclonal antibodies (mAb) against ICAM-1 (R&D Systems). After washing cell pellets twice in buffer the FITC-conjugated goat-anti-mouse IgG (Becton Dickinson, Heidelberg, Germany) was added for 30 min. Fluorescence intensities were measured by flow cytometry (FACScan, Becton Dickinson). Data collected from 1×10^4 cells were analyzed using "CellQuest" software. All experiments were repeated at least three times.

RT-PCR

Total RNA was isolated from mock-infected or AD169-infected cells using TRIZOL according to the manufacturer's instructions (Gibco-BRL Life Techologies, Gaithersburg, Md.). RNA was reverse transcribed using random hexamer priming. One microgram of total RNA was denatured at 70°C for 10 min and chilled on ice. The denatured RNA was then co-incubated with 2.5 μM random hexamer oligonucleotides, 1 μM of each dNTP, 5 mM $MgCl_2$, 1 μl RNAse inhibitor (Boehringer Mannheim), and 1 μl MuLV reverse transcriptase (Gibco-BRL) in 1 × PCR buffer II (Perkin-Elmer Corp.) for 1 hr at 37°C. The reverse transcription was inactivated for 5 min at 95°C before amplification. ICAM-1 primers used were 5′-GTG ACA TGC AGC ACC TCC TG -3′ (position 148–167) and 5′-TCC ATG GTG ATC TCT CCT CA -3′ (position 555–536) [Wakita et al., 1996], IL-8 primers used were 5′-TCT CTT GGC AGC CTT CCT GAT -3′ (position 126–146) and 5′-TTT CTG TGT TGG CGC AGT GTG -3′ (position 278–298). IL-8 primers were selected according to the sequence information of Mukaida et al. [1989]. The sequence of GAPDH primers used as control were as follows 5′-TGG GGA AGG TGA AGG TCG GA-3′ (position 61–81) and 5′-GAA GGG GTC ATT GAT GGC AA-3′ (position 151–171) [Bordow et al., 1994]. PCR amplification of the cDNA was carried out by adding 0.5 μg Taq DNA polymerase (Boehringer Mannheim). PCR amplification was done using 26 cycles in a DNA thermocycler (Perkin Elmer Corp.) in the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 52°C, and extension for 1.5 min at 72°C. PCR products were resolved alongside DNA marker on an 8% polyacrylamide gel, stained with silver nitrate and photographed. The photographs were analyzed further by scanning densitometry using E.A.S.Y. RH system (HeroLab, Wiesloch, Germany), and the ratio of IL-8 or ICAM-1/GAPDH band intensity was calculated.

Migration Assay

Granulocytes were isolated from peripheral blood of healthy donors via density gradient centrifugation (Polymorphprep, Nycodens). Granulocytes/ml (5×10^6) were placed in the upper compartment of Boyden chambers with a pore size of 3 μm (Becton Dickinson). Supernatants of the respective HFF cultures were transferred to the lower compartment. For positive control purposes, MEM containing *n*-formyl-met-leu-phe (FMLP; Sigma; 1×10^{-7} M) was used. All migration assays were carried out in 6 well plates. The number of cells that migrated into the lower compartment and adhered to the bottom was determined by counting after 30 min.

TABLE I. Effects of Anti-CMV Drugs on IEA, LA Expression, and Infectious Virus Production

Drug concentration (μM)	% of HCMV positive cells		Log virus titre (I.E.F.U./ml)
	IEA	LA	
0	99.5 ± 0.41	99.1 ± 0.26	4.98 ± 0.035
GCV			
5	99.8 ± 0.15	38.2 ± 5.7	4.61 ± 0.042
10	99.6 ± 0.26	4.9 ± 0.85	1.99 ± 0.039
40	99.9	0	0
PFA			
25	99.6 ± 0.26	46.7 ± 6.2	4.32 ± 0.52
50	99.7 ± 0.35	12.1 ± 1.9	2.12 ± 0.47
200	99.3 ± 0.43	0	0
HPMPC			
0.2	99.6 ± 0.26	61.3 ± 7.4	4.59 ± 0.038
0.8	99.8 ± 0.1	4.2 ± 0.63	1.87 ± 0.049
2.0	99.7 ± 0.2	0	0
ISIS 2922			
0.1	68.5 ± 7.3	71.5 ± 0.72	4.73 ± 0.034
0.4	9.8 ± 1.2	11.5 ± 1.8	2.82 ± 0.048
1.0	0.21 ± 0.043	0.15 ± 0.011	0.61 ± 0.021
ISIS 26062			
0.1	99.6 ± 0.26	96.5 ± 1.8	4.75 ± 0.043
0.4	98.5 ± 0.53	97.4 ± 0.62	4.81 ± 0.037
1.0	94.1 ± 0.93	96.2 ± 2.1	4.69 ± 0.036

Statistical Analyses

Determination of statistical significance was carried out with the Student's *t*-test. Data groups were considered significantly different when $P < 0.05$.

RESULTS

Antiviral Assays

The effects of different antiviral drugs used for the treatment of patients with HCMV disease were evaluated in a cell culture model with human permissive fibroblasts. The expression of HCMV specific IE and L antigens and the production of infectious viruses was measured (Table I). The inhibitors of virus DNA replication GCV, HPMPC and PFA completely inhibited both LA and the production of infectious virus particles in a concentration dependent manner but had no effects on IEA expression. In contrast, ISIS 2922 not only inhibited LA and infectious virus production but also IEA expression (Table I, Fig. 1); however, the drug was not able to inhibit completely virus replication as demonstrated by the detection of low amounts of cells expressing IEA, LA and of infectious virus in cultures treated with 1 μM ISIS 2922 (Table I). ISIS 2922 reduced the number of cells expressing both IEA and LA by more than 99%. The infectious virus titre was reduced by more than 10^4-fold. Concentrations as high as 10 μM completely inhibited expression of virus antigens and the production of infectious virus. Concentrations above 1 μM, however, were not used in further experiments because pretreatment of cells with these concentrations was previously shown to influence virus infection in a nonspecific manner [Azad et al., 1993; Anderson et al., 1996]. ISIS 26062 (a nonspecific control oligonucleotide) at concentrations ranging from 0.1–1 μM impaired neither the expression of HCMV IEA and LA nor the production of infectious virus.

Effects of HCMV on IL-8 Expression

The influence of HCMV infection (MOI 1) on the secretion of IL-8 was measured by ELISA in the supernatants of fibroblast cultures at various time points post infection. It could be revealed that HCMV significantly augmented IL-8 secretion already 4 hr after infection (Fig. 2). At that time point 4-fold higher levels of IL -8 in HCMV-infected cultures were measured relative to mock-infected cells (1.93 ± 0.28 ng/10^6 cells; vs. 0.48 ± 0.071 ng/10^6 cells; $P < 0.05$). Time kinetics showed that the levels of IL-8 in mock-infected cultures were slightly increased (1.76 ± 0.24 ng/10^6 cells and 1.55 ± 0.19 ng/10^6 cells) 24 hr and 72 hr post infection, respectively. HCMV-infected cultures produced 7- to 9-fold higher amounts (i.e., 12.3 ± 1.9 ng/10^6 cells and 13.8 ± 1.7 ng/10^6 cells, both $P < 0.05$) of IL-8 relative to the mock-infected cultures 24 hr and 72 hr post infection, respectively. The results shown in Figure 2 also suggest that these effects depend on virus replication because neither UV-inactivated virus nor virus-free supernatants collected from infected cultures increased amounts of IL-8.

Effects of HCMV on ICAM-1 Expression

ICAM-1 expression was analyzed by means of flow cytometry. HCMV-infected fibroblast cultures (MOI 1) exhibited 1.8-fold increase of cell surface ICAM-1 expression relative to the mock-infected cells already 4 hr post infection (95 ± 12 FIU vs. 173 ± 29 FIU; $P < 0.05$) (Fig. 2). After 24 and 72 hr post infection ICAM-1 expression was found to be further elevated 3- and 4-fold, respectively. Similar as for IL-8, the treatment of the cells with UV-inactivated virus or virus-free supernatant from infected cultures did not lead to upregulation of ICAM-1.

Effects of Antiviral Drugs on HCMV-Induced Upregulation of IL-8 and ICAM-1

The ability of antiviral drugs to influence HCMV-induced upregulation of IL-8 secretion and cell surface expression of ICAM-1 was studied 72 hr post infection in cultures treated continuously with different drugs that were added to the culture medium both before and after virus infection as described in the methods section. As shown in Figure 3 GCV, HPMPC and PFA influenced neither IL-8 secretion nor ICAM-1 expression in infected cells although virus replication was completely inhibited. In contrast, 1 μM ISIS 2922 (inhibiting about 99% of both IEA- and LA expression) reduced HCMV-induced production of IL-8 3-fold ($P < 0.05$). The amount of this chemokine, however, was still 3-fold higher than in supernatants of mock-infected cultures (105 ± 17 FIU vs. 315 ± 42 FIU).

Effects of HCMV and Antiviral Drugs on IL-8 and ICAM-1 mRNA Levels

To find out whether HCMV-IE augments IL-8 and ICAM-1 expression at the transcriptional level RT PCR

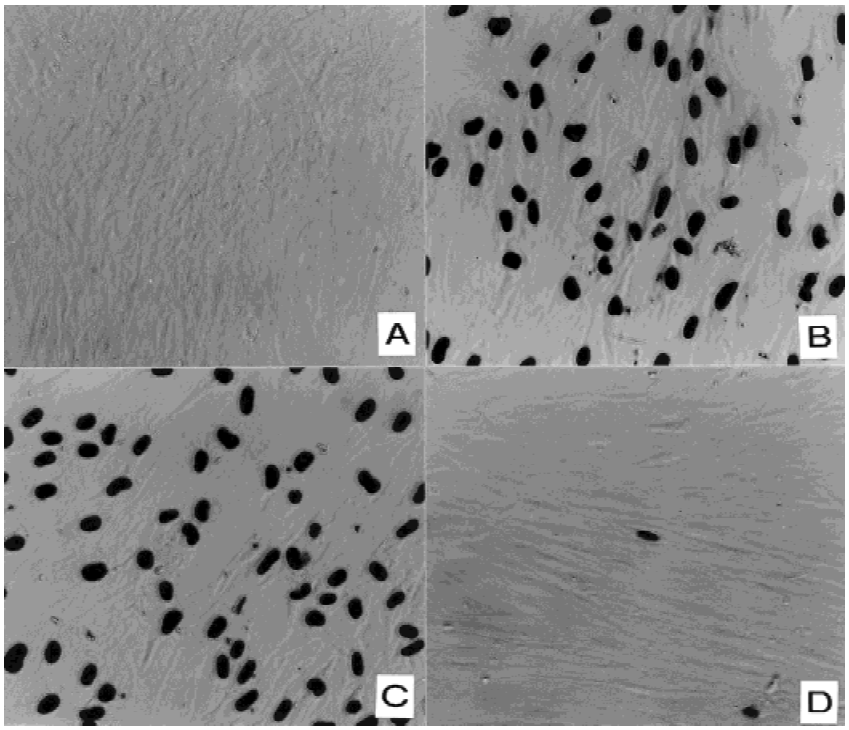

Fig. 1. Photomicrographs of mock-infected (**A**) and HCMV-infected (**B**–**D**) HFF cultures stained by means of immunoperoxidase (24 hr after infection) using Mab810 antibody that recognizes HCMV IE nuclear antigens. Infected cells were otherwise untreated (B), treated with ganciclovir (C) or with ISIS 2922 (D).

was performed 4, 24, and 72 hr after infection. In addition, we measured effects of antiviral drugs including ISIS 2922 and GCV on mRNA expression of the inflammatory molecules in mock- and AD169-infected HFF (Figs. 4 and 5). Silver nitrate staining of the IL-8 PCR products from untreated HFF and HCMV-infected cells are shown in Figure 4. The density of each band was measured by densitometric analysis and compared with GAPDH. IL-8/GAPDH ratios are indicated as relative IL-8 mRNA expression and the 4 h mock-infected values were defined as 1. The data reveal that the constitutive mRNA expression of IL-8 declines from 4–72 hr after mock infection (1.0 ± 0.08, 0.3 ± 0.05, 0.15 ± 0.02) whereas HCMV-infected cells exhibit 2.1-fold elevated levels at 4 hr (mock-infected: 1.0 ± 0.08 vs. HCMV-infected: 2.1 ± 0.24), 12-fold elevated levels 24 hr after infection (0.3 ± 0.05 vs. 3.6 ± 0.31), and 9-fold after 72 hr (0.15 ± 0.02 vs. 1.35 ± 0.14; Fig. 4D). Ganciclovir did not impair HCMV-induced IL-8 mRNA expression 4 hr, 24 hr, and 72 hr after infection (2.3-fold, 13-fold, and 10-fold, respectively; Fig. 4E). In contrast, ISIS 2922 significantly suppressed HCMV-induced IL-8 mRNA expression (Fig. 4F). Four hours after infection HCMV-induced IL-8 mRNA expression was only 1.6-fold (2.1-fold without ISIS 2922) compared with mock-infected control. 24 hr and 72 hr after infection the virus-mediated IL-8 mRNA expression was significantly reduced by ISIS 2922 (4.4-fold and 1.5-fold, respectively) when compared with infected cells without ISIS 2922 (12-fold and 9-fold; $P < 0.05$).

The basal expression of ICAM-1 was shown to be elevated during the measured time course (4–72 hr after infection). HCMV infection resulted in 4.9-fold (1.0 ± 0.13 vs. 4.9 ± 0.5) expression of ICAM-1 mRNA 4 hr and 2.5-fold (2.0 ± 0.21 vs. 5.0 ± 0.43) 24 hr post infection whereas similar levels of the specific mRNA were observed both in mock- and HCMV-infected cells 72 hr post infection (3.2 ± 0.23 vs. 3.3 ± 0.36; Fig. 5D). Similar to the findings with IL-8 mRNA, ganciclovir had no relevant influence on the HCMV-induced expression of ICAM-1 (Fig. 5E) whereas ISIS 2922 almost completely inhibited HCMV-induced effects on ICAM-1 expression ($P < 0.05$) at 4 hr and 24 hr (mock-infected: 1.0 ± 0.08 vs. HCMV-infected plus ISIS 2922: 1.08 ± 0.06; 2.4 ± 0.3 vs. 2.1 ± 0.19, respectively; Fig. 5F).

Effects of Antiviral Drugs on Chemoattractant Activity

We further tested whether the observed reduction of IL-8 levels by ISIS 2922 may also result in reduced chemoattraction of neutrophils. Therefore, supernatants from the respective cultures (72 hr after onset) were transferred into the lower compartments and neutrophils in the upper compartments of Boyden chambers. In three independent experiments, activity of cell migration was assessed by counting the adherent cells (cells/mm^2) in the lower compartment after 30 min. As depicted in Figure 6 supernatants from HCMV-infected HFF attracted more than 3-fold numbers of neutrophils compared with supernatants from HFF alone (296 ± 20 and 84 ± 12, respectively).

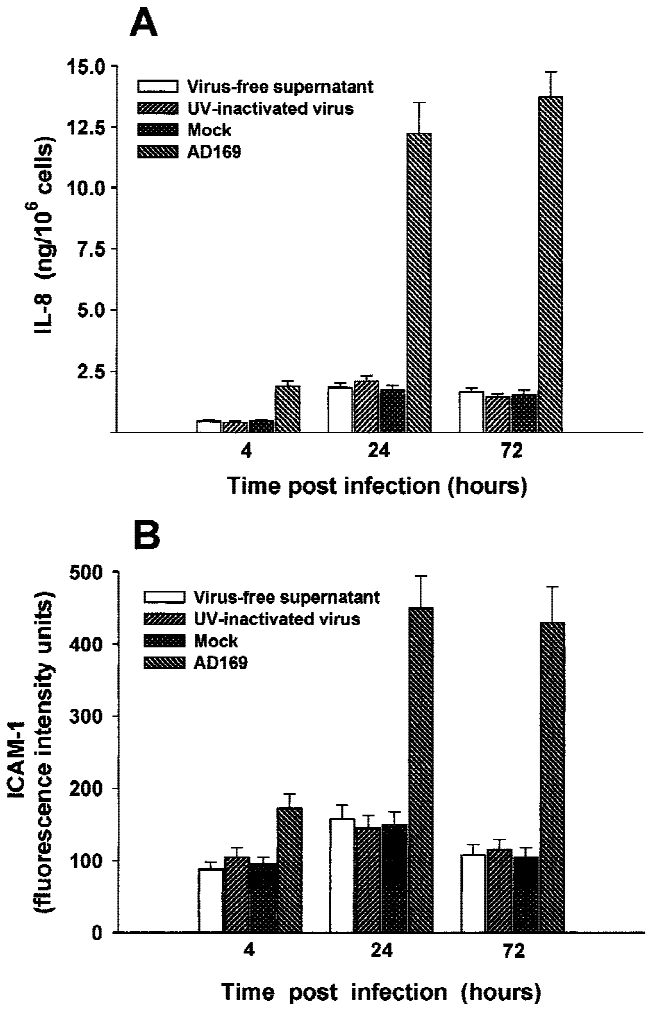

Fig. 2. Time course of IL-8 secretion (**A**) as determined by means of ELISA and of ICAM-1 expression (**B**) by means of flow cytometry 4, 24, and 72 hr after infection. Data are depicted as means ± SD from one representative experiment performed in triplicates.

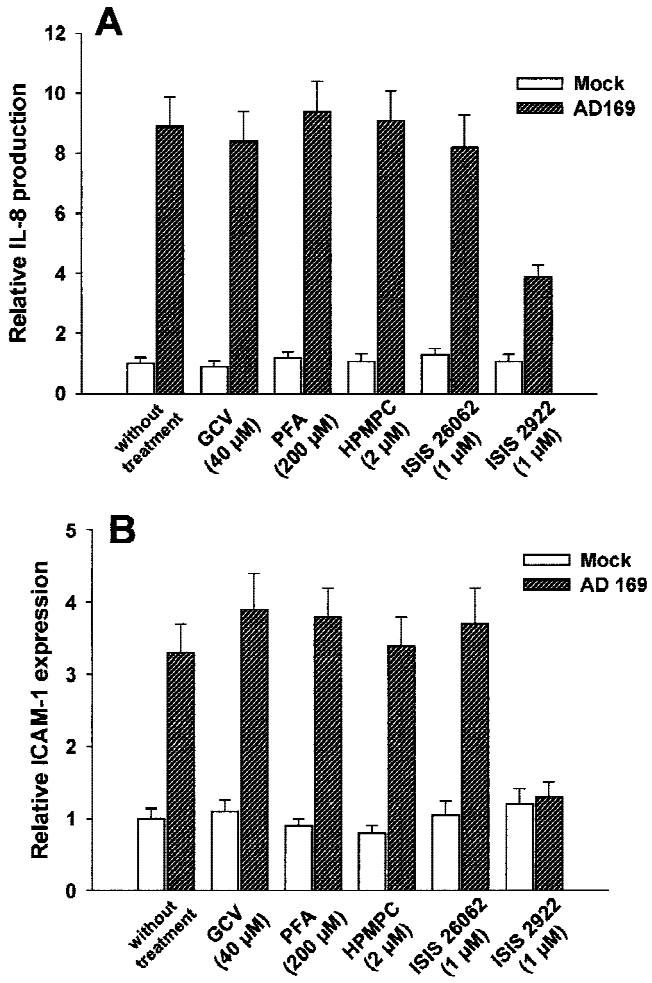

Fig. 3. Effects of antiviral drugs on HCMV-induced IL-8 production (**A**) and ICAM-1 expression (**B**) as determined by ELISA and flow cytometry, respectively, 72 hr after infection. Data relative to the mock-infected control are depicted as means ± SD from one representative experiment performed in triplicates.

Whereas GCV had no effect on the HCMV-induced chemoattraction ISIS 2922 significantly inhibited HCMV-induced neutrophil migration (304 ± 28 and 168 ± 24, respectively).

DISCUSSION

There is growing evidence that in immunocompromised patients HCMV disease has an immunopathological component that results in clinically overt signs and symptoms of inflammation. For example, in patients with allogeneic bone marrow transplantation allogeneic immune responses rather than direct viral cytopathogenicity might be responsible for HCMV pneumonitis [Grundy et al., 1987; Fishman and Rubin, 1998]. In solid organ transplantation HCMV infection is frequently associated with graft rejection crises [von Willebrand et al., 1986; Pouteil Noble et al., 1993; Lautenschlager et al., 1997]. It is thought that HCMV may induce inflammatory mechanisms and in turn inflammation may induce HCMV replication. The role of T cell-mediated immunity in HCMV pathogenesis has recently also be shown in AIDS patients with HCMV retinitis. In these patients vitreitis probably resulted from restoration of specific immune reactivity against smoldering HCMV infection after onset of highly active antiretroviral therapy (HAART) [Jacobson et al., 1997; Mitchel et al., 1997; Karavellas et al., 1999].

It has been assumed earlier that HCMV-infected cells may secrete chemoattractants and exhibit modified adhesion molecule expression, thus attracting leukocytes toward the site of infection. Indeed, it could be shown that HCMV modifies the expression of various relevant immune surface membrane molecules such as HLA class I and class II and ICAM-1 [Scholz et al., 1992; Grundy and Downes, 1993; Sedmak et al., 1994; Waldman and Knight, 1996]. Moreover, it was found that HCMV infection of cultured endothelial cells leads to enhanced secretion of the C-X-C chemokines IL-8 and GRO-α [Craigen et al., 1997; Scholz et al., 1998]. This HCMV-induced enhancement of chemokine secretion could be important for virus transmission from

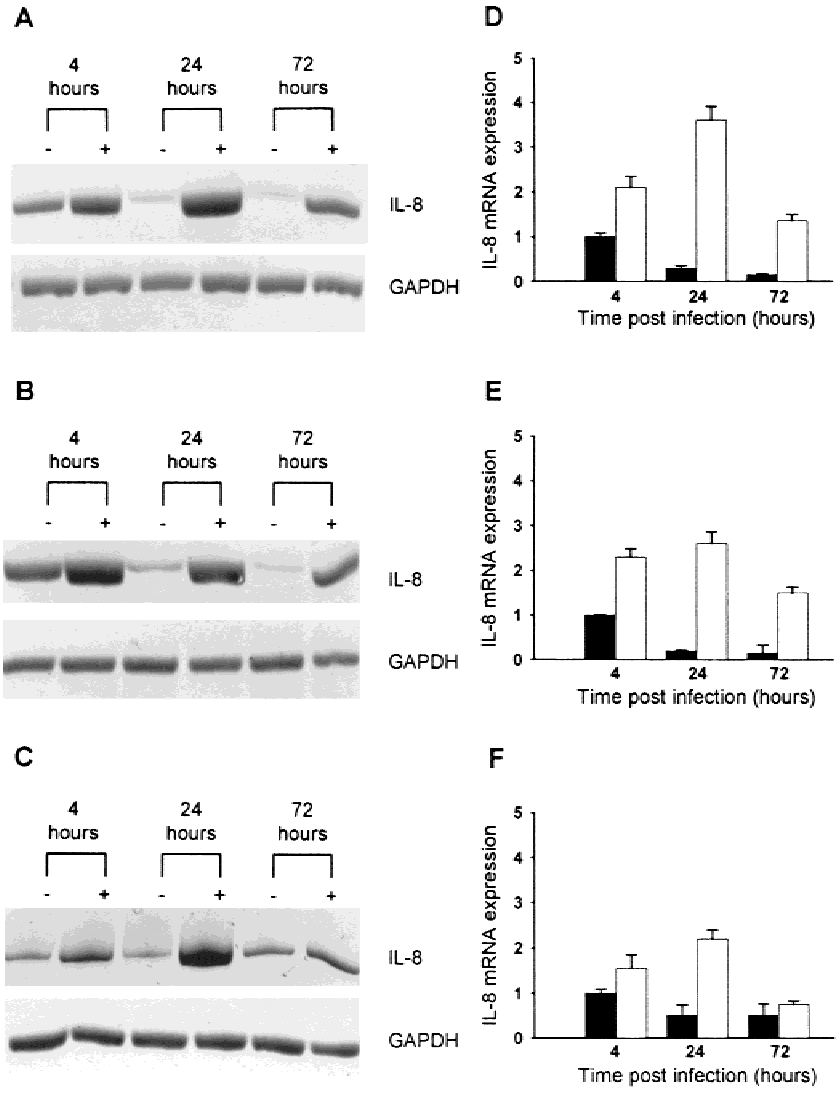

Fig. 4. Time course of IL-8 and GAPDH mRNA expression in mock-infected (–, black bars) and HCMV-infected (+, white bars) cells as determined by RT PCR. PCR products were stained with silver nitrate 4, 24, and 72 hr after infection (**A**–**C**). Densitometric analysis of the bands was performed and the ratio between IL-8 specific bands and GAPDH bands was calculated (**D**–**F**). The value of the mock-infected control at 4 hr was defined as 1. All other mRNA expression levels are depicted as the mean of three experiments ± SD relative to the 4 hr mock-infected control. Untreated cells: A, D; Ganciclovir-treated cells: B, E; ISIS 2922-treated cells: C, F.

endothelial cells to neutrophils that might also play an important role in carrying HCMV from the infected endothelium into the tissue. Attraction of neutrophils and monocytes by HCMV-infected endothelial cells and the acquisition of infectious HCMV by neutrophils/monocytes from infected endothelial cells were shown in vitro [Waldman et al., 1995; Grundy et al., 1998].

Proinflammatory cytokines such as TNF-α and IL-1, lipopolysaccharide, and mitogens but also IL-8 strongly stimulate the secretion of other chemokines [Anisowicz et al., 1991; Dezube et al., 1992]. These cytokines/chemokines are discussed to be partly involved in HCMV infection. For example, TNF-α and IL-8 are suspected to stimulate HCMV replication [Capobianchi et al., 1997; Prösch et al., 1995]. In turn, HCMV infection upregulates TNF-α and IL-8 production in vitro [Murayama et al., 1994; Pulliam et al., 1995; Craigen et al., 1997]. It has been found in vivo, that elevated serum cytokines are associated with cytomegalovirus infection and disease in bone marrow transplant recipients [Humar et al., 1999].

From this knowledge, it is conceivable that this modified balance between inflammatory cytokines, chemokines and adhesion molecules may contribute to severe tissue damage. Regarding the treatment of HCMV disease account should be taken that a) proinflammatory proteins may be upregulated due to transcriptional effects of viral gene products (restricted to infected cells) or b) HCMV-induced cytokines influence uninfected bystander cells in an unspecific manner. Therefore, treatment of HCMV disease with inhibitors of DNA synthesis (i.e., GCV, PFA or HPMPC) may be of limited benefit when the disease is predominantly based on immunopathological mechanisms. An effec-

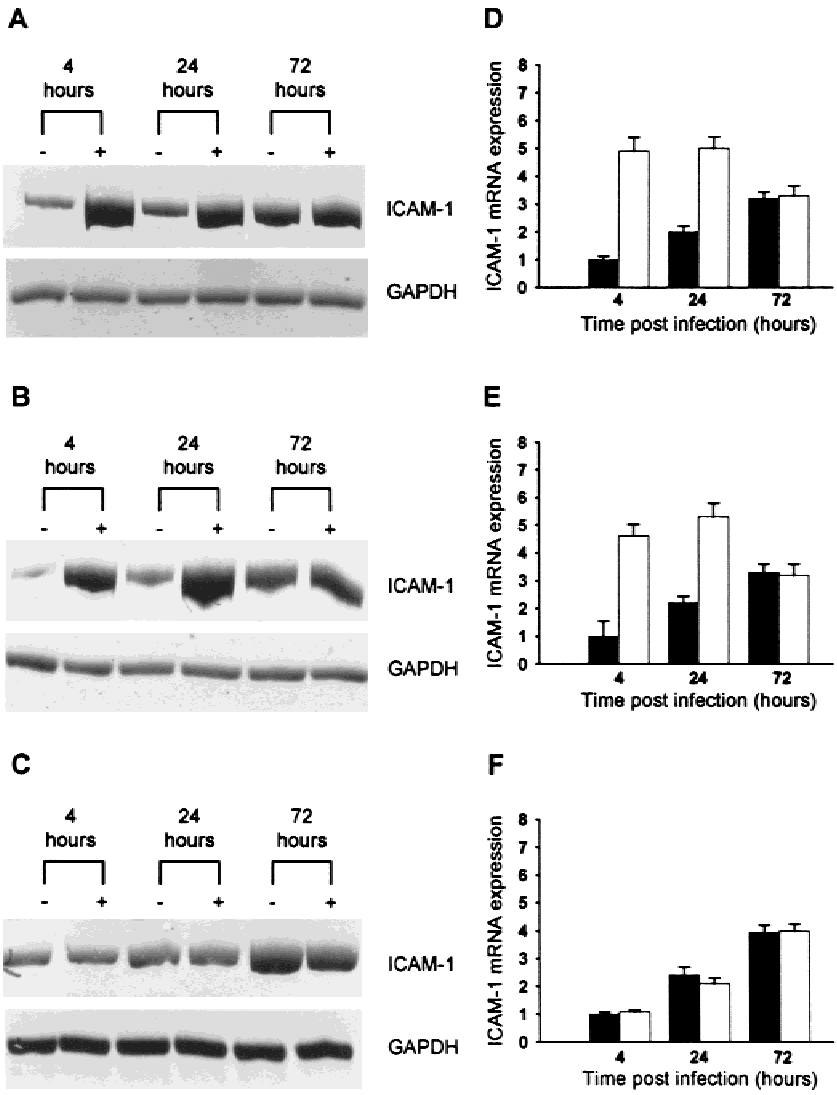

Fig. 5. Time course of ICAM-1 and GAPDH mRNA expression in mock-infected (–, black bars) and HCMV-infected (+, white bars) cells as determined by RT PCR. PCR products were stained with silver nitrate 4, 24, and 72 hr after infection (**A**–**C**). Densitometric analysis of the bands was performed and the ratio between ICAM-1 specific bands and GAPDH bands was calculated (**D**–**F**). The value of the mock-infected control at 4 hr was defined as 1. All other mRNA expression levels are depicted as the mean of three experiments ± SD relative to the 4 hr mock-infected control. Untreated cells: A, D; Ganciclovir-treated cells: B, E; ISIS 2922-treated cells: C, F.

tive treatment of HCMV disease should cover both, inhibition of virus replication and prevention of HCMV-induced cell-mediated immune responses resulting in inflammation.

Craigen and Grundy [1996] demonstrated that HCMV-induced upregulation of cell surface adhesion molecules including ICAM-1 and LFA-3 in cultured fibroblasts can not be prevented by treatment with inhibitors of HCMV DNA synthesis (GCV, PFA). Similarly, in our previous experiments GCV and PFA did not prevent HCMV-induced upregulation of several C-C and C-X-C chemokines after infection of cultured human retinal pigment epithelial and glial cells [Scholz et al., 1998]. In the present study with human fibroblasts we included cidofovir (HPMPC), a novel inhibitor of HCMV DNA replication [Neyts et al., 1991; Naesens et al., 1997]. HPMPC, like GCV and PFA failed to inhibit HCMV-induced upregulation of ICAM-1 and secretion and functional activity of IL-8. These findings indicate that the currently available drugs used for systemic treatment of HCMV disease fail to prevent HCMV-induced immunomodulation.

It has been demonstrated that virus adsorption and penetration before HCMV gene expression may stimulate cellular metabolism [Boldogh et al., 1993; Yurochko et el., 1997]. In our experiments, however, UV-irradiated HCMV enhanced neither IL-8 nor ICAM-1 expression suggesting that de novo synthesis of virally encoded proteins is required for the observed effects. HCMV IE proteins are known to transactivate numerous cellular genes [Monick et al., 1992; Hagemeier et al., 1994; Mocarski, 1996; Murayama et al., 1997; Yurochko et al., 1997; Cinatl et al., 1999]. These transactivating properties of HCMV IE may account for the ob-

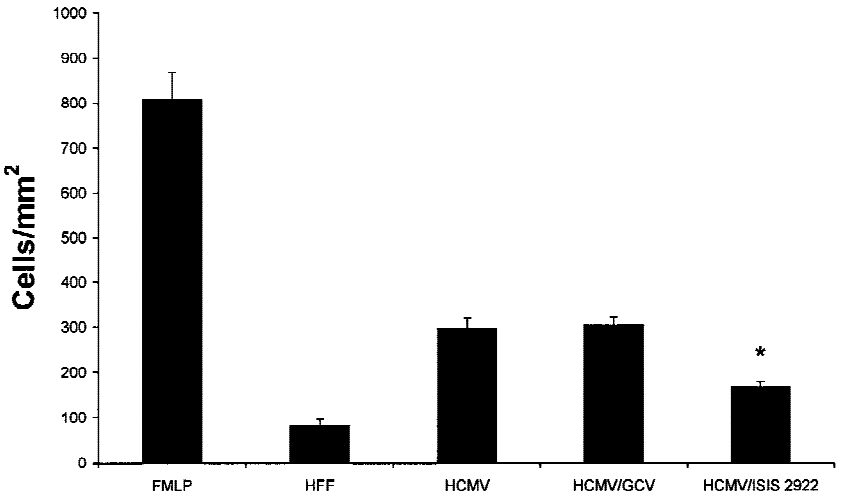

Fig. 6. Effects of cell culture supernatants (72 hr after infection) on neutrophil migration in Boyden chambers. Cells that migrated from the upper to the lower chamber and adhered to the bottom of the culture dish were counted (cells/mm^2). The lower chamber contained either a potent chemoattractant (FMLP) as a positive control, supernatants from mock-infected HFF cells (HFF), supernatants from HCMV-infected cells (HCMV), supernatants from HCMV-infected and ganciclovir-treated cells (HCMV/GCV), or supernatants from HCMV-infected and ISIS 2922-treated cells (HCMV/ISIS 2922). Data are depicted as means ± SD from one representative experiment performed in triplicates. *HCMV-induced migration was significantly reduced by ISIS 2922.

served increased ICAM-1 and IL-8 gene expression. In the human monocytic cell line THP-1 HCMV-induced IL-8 gene transcription was dependent on the activation of AP-1 and NF-B transcription factors [Murayama et al., 1997]. Thus, HCMV proteins may indirectly promote IL-8 gene expression. In another study, evidence has been obtained that ICAM-1 upregulation in cultured endothelial cells may be due to direct interaction of HCMV IE gene products with ICAM-1 promotor elements [Burns et al., 1999].

ISIS 2922 has been developed for intravitreal treatment of HCMV retinitis and provides a novel therapeutic option for direct inhibition of IE gene expression and in addition allows the experimental assessment of the role of IE proteins in influencing the activity of cellular gene transcription [Cinatl et al., 1999]. ISIS 2922 is a phosphorothioate oligonucleotide complementary to the mRNA encoding the major polypeptide products of IE region 2 (IE2). [Azad et al., 1993; Anderson et al., 1996;]. Previous studies demonstrate that treatment of normal human fibroblast cells with ISIS 2922 before infection with HCMV results in a sequence-dependent reduction in the levels not only of IE2 but also of IE1 [Anderson et al., 1996; Cinatl et al., 1999]. Therefore, it can not be excluded that some mechanisms such as inhibition of HCMV adsorption or penetration accounts for antiviral and anti-inflammatory effects of the drug; however, such nonspecific antiviral effects were previously observed at concentrations of ISIS 2922 higher than 1 μM [Anderson et al., 1996].

In this study ISIS 2922 has been shown to inhibit HCMV-induced ICAM-1 and IL-8 expression on the transcriptional and protein level. ISIS 2922, however, did not completely inhibit IL-8 expression. This seems to correlate with the functional migration studies in which the number of migrated neutrophils was only reduced to approximately 50%. These findings suggest that residual effects of HCMV on cellular metabolism occur despite the presence of the drug. This could result from the inability of ISIS 2922 to completely inhibit IE gene expression resulting in transactivation of the IL-8 gene either directly or through the activity of cellular transcription factors. In this regard, it should be tested whether additional or other strategies such as suppression of transcription factors may further help to suppress HCMV-induced upregulation of IL-8.

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